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# INTERACTIONS OF VARIOUS AIR POLLUTANTS ON CAUSATION OF PULMONARY DISEASE

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IIT Research Institute

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This report consists of four studies. The materials, methods, and experimental protocol for each study were presented. The parameters of interest were hemagglutination-inhibition (HI) and serum neutralization (SN) antibody formation, serum immunoglobulin levels, lung histopathology, mortality rates, lung lesion scores, and extent of lung edema in mice. The four studies were: (1) immune response in mice during long term exposure to nitrogen dioxide, (2) immune response in mice during a four-week exposure to $NO_2$, (3) effect of chronic exposure to $NO_2$ on resistance to Klebsiella pneumoniae, and (4) effects of manganese on resistance to respiratory infection. In the first study prior to vaccination the mice were exposed continously to 2ppm $NO_2$, 0.5ppm $NO_2$ with 1-hr daily peaks of 2ppm $NO_2$ 5 days in a week, or filtered air for 3 months. They were thereafter, held in either an $NO_2$ environment or filtered air. At specified time intervals, groups of mice were challenged by the respiratory route with live influenza virus. The same $NO_2$ levels were utilized in the second and third study. The $NO_2$ exposure time was different for each study. The morality rates and results of measured parameters are evaluated and conclusions are presented for each study.

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Immunoglobulin
Mortality rates
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## FOREWORD

This is Report No. IITRI-L6069-4 (Final Report), entitled, "Interaction of Various Air Pollutants on Causation of Pulmonary Disease", Contract No. 68-02-0666, IITRI Project L6069. The studies were conducted by IIT Research Institute for the Environmental Protection Agency during the period from August 18, 1972 through August 17, 1973.

The principal investigator is Dr. Richard Ehrlich, the co-investigator is Dr. James D. Fenters and the principal professional associate is Dr. Robert Z. Maigetter. Other personnel participating in the program are Dr. C. D. Port, Mr. J. C. Findlay, Mr. T. Sharp, Ms. E. Silverstein and Mr. S. Britton.

The experimental data are recorded in IITRI Logbooks C20951, C21056, C21281, C11790, C15809, C21474, C21475, D1756, D1812 and D1823.

Respectfully submitted,

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TABLE OF CONTENTS


| | | Page |
|---|---|---|
| I. | IMMUNE RESPONSE IN MICE DURING LONG-TERM EXPOSURE TO NITROGEN DIOXIDE | I-1 |
| | A. Introduction | I-1 |
| | B. Materials and Methods | I-1 |
| | C. Results | I-7 |
| | 1. Body Weights | I-7 |
| | 2. Serology | I-7 |
| | 3. Immunoglobulins | I-9 |
| | 4. Infectious Challenge | I-16 |
| | a. Four Weeks after Vaccination | I-17 |
| | b. Sixteen Weeks after Vaccination | I-20 |
| | c. Twenty-eight Weeks after Vaccination | I-20 |
| | 5. Lung Edema | I-21 |
| | 6. Histopathology | I-21 |
| | D. Summary | I-27 |
| II. | IMMUNE RESPONSE IN MICE DURING A 4-WEEK EXPOSURE TO $NO_2$ | II-1 |
| | A. Introduction | II-1 |
| | B. Materials and Methods | II-1 |
| | C. Results | II-5 |
| III. | EFFECT OF CHRONIC EXPOSURE TO $NO_2$ ON RESISTANCE TO K. PNEUMONIAE | III-1 |
| | A. Introduction | III-1 |
| | B. Materials and Methods | III-1 |
| | C. Results | III-3 |
| IV. | EFFECTS OF MANGANESE ON RESISTANCE TO RESPIRATORY INFECTION | IV-1 |
| | A. Introduction | IV-1 |
| | B. Materials and Methods | IV-1 |
| | C. Results | IV-4 |

# I. IMMUNE RESPONSE IN MICE DURING LONG-TERM EXPOSURE TO NITROGEN DIOXIDE

## A. Introduction

Previous studies conducted in our laboratories indicated that chronic exposure to 1 or 5 ppm of nitrogen dioxide ($NO_2$) markedly affected the ability of squirrel monkeys to produce serum neutralization antibodies (Fenters, et al. Am. Rev. Resp. Dis. 104, 448, 1971; Fenters, et al. Arch. Environ. Health 27, 85, 1973). To elucidate the effect of chronic exposure to low concentrations of $NO_2$ on the immunological response, further studies were conducted in mice vaccinated with a highly purified influenza virus vaccine. Parameters of interest were hemagglutination-inhibition (HI) and serum neutralization (SN) antibody formation, serum immunoglobulin levels, lung histopathology, and mortality rates, lung lesion scores, and extent of lung edema in mice challenged with live infectious influenza virus.

## B. Materials and Methods

Animals. Four-week-old specific-pathogen-free male Swiss albino mice, CD-1 strain, were obtained from Charles River Laboratories. After a two-week quarantine period, the mice were placed in the environmental chambers and held for two days before initiation of the exposures. During the exposures, the mice were removed from the chamber for one hour three times a week for maintenance. Clean cages were provided once a week and food and water were provided ad libitum.

Influenza Virus. Mouse-adapted influenza $A_2$/Taiwan/1/64 virus was passaged several times in mice and 20% lung suspension of the virus was used for all infectious challenges. Prior to use, the virus was identified by use of specific antiserum obtained from the National Institutes of Health.

Vaccine. Chick embryo $A_2$/Taiwan/1/64 influenza vaccine (Zonomune), Lot No. BP0549, was supplied by Eli Lilly and Company, Indianapolis. Mice were vaccinated by a single subcutaneous injection of approximately 279 CCA units in 0.1 ml vaccine.

HI and SN Titrations. Tests were performed in duplicate, by the microtiter method in disposable V-plates (Cooke Engineering Co., Alexandria, Va.) described by Davenport and Minuse (Diagnostic Procedures for Viral and Rickettsial Diseases, 3rd ed. Am. Public Health Assoc., New York, 1964). In all tests, 1% chicken red blood cells were used.

Four hemagglutinating units of antigen were used for the HI test. All antisera were heat-inactivated at 56°C for 30 min and treated with trypsin-periodate to remove nonspecific inhibitors of hemagglutination.

The protocol used for the test was similar to that described in the USPHS Requirements (Division of Biologics Standards, 6th rev., 1947). Sera were heat-inactivated at 56°C for 30 min, serially diluted, and incubated with an equal volume of influenza virus for 1 hr at 4°C. The serum-virus mixture was then tested in 10-day-old embryonated chicken eggs. The eggs were inoculated by the allantoic route with 0.1 ml of the virus-serum mixture, incubated at 37°C and harvested when an $EID_{50}$ (50% egg infectious dose) dose of 32 to 320 was attained as indicated by hemagglutination of the virus control. The $EID_{50}$ dose was determined by parallel infectivity tests in eggs by using a 0.1-ml virus-saline mixture. Phosphate buffered saline (PBS), PBS plus normal mouse serum, and normal mouse serum plus virus were inoculated into eggs as controls.

Immunoglobulin Concentration. Quantitative radial immunodiffusion plates for mouse immunoglobulins IgA, $IgG_1$, $IgG_2$, and IgM were procured from Meloy Laboratories, Inc., Springfield, Virginia. Reference standards obtained from pooled sera of normal mice were assayed daily, in duplicate, to provide quality control.

Duplicate serum samples were placed in preformed wells, the plates incubated at 22°C for 18 hr and the radial diffusion diameters measured using a Bausch & Lomb 7x lens. Duplicate radial diffusion diameters were recorded for each of the pooled serum sample; consisting of seven to 10 pooled samples at each experimental point.

Nitrogen Dioxide Exposure. To maintain the control and experimental mice under similar conditions, three identical aluminum-lined chambers (4 x 6 x 6.5 ft) were used for the exposures. Randomly selected mice were housed in suspended wire cages, which were periodically rotated to various positions on the cage racks. This assured a thorough and unbiased exposure to the experimental environment. A minute amount of $NO_2$ was continuously passed from a cylinder through a stainless steel tube into a glass mixing vessel where it was diluted and mixed with charcoal-filtered ambient air. The mixture was then passed into the $NO_2$ exposure chambers at a rate of 20 changes per hour. The same air flow pattern was maintained in the control chamber where the chaircoal-filtered ambient air was used. To verify the homogeneity of $NO_2$, air samples were taken from different sections of the chambers and the $NO_2$ concentration determined and calculated by the Saltzman method. A Mast $NO_2$ gas analyzer was used for continuous monitoring. The mean temperature in the chambers was 24° $\pm$ 1°C.

Scoring Pulmonary Lesions. The extent of pulmonary lesions was expressed as the percentage of the total lung consolidated. A score of 1 represented 25% lung consolidation, 2 = 50%, 3 = 75%, 4 = 100%, and a score of 5 represented animals that died during the experiment.

Lung Edema. The ratio of the wet-to-dry lung weight of lungs was used to express the extent of edema. Three pools of three to five lungs each were used for each experimental group. The pooled lungs were weighed immediately after removal from the mice then dried in a vacuum desiccator and reweighed at 24-hr intervals until there was no additional weight loss.

Aerosol Challenge. Infectious respiratory challenge was conducted in a 350-liter plastic aerosol chamber (60 x 60 x 95 cm) installed within a microbiological safety cabinet. A University of Chicago Toxicity Laboratory type atomizer was used to produce a viral aerosol with a majority of <5μ-mass-median-diameter airborne particles. The microbial suspensions were fed to the atomizer by a 50-ml syringe activated by a motor-driven piston delivering the suspension at a rate of 0.4 ml/min. Filtered air was supplied to primary and secondary inlets of the atomizer at a flow rate of 33 liters/min. The chamber was maintained at 78 ± 6% RH and 24° ± 1°C.

The aerosol was sampled with an all-glass impinger (AGI-30) containing PBS with 0.2% bovine serum albumin as a collecting fluid. The inhaled dose was estimated on the basis of the concentration of the virus per liter of air, respiratory minute volume of the mice, and the duration of exposure to the aerosol.

For the infectious challenge, groups of mice were placed in the chamber and exposed to the aerosol for 5 to 6 min. After the challenge, the mice were air-washed for 10 min, removed from the aerosol chamber and held for 14 days in filter-capped cages in a clean-air, isolated animal room.

Histopathology. The mice were anesthetized with carbon dioxide, the lungs and heart removed as a unit, and representative tissues were fixed in a 10% phosphate buffered formalin solution. After blocking in paraffin, 4μ-thick sections were cut in a rotary microtome, stained with hematoxylin and eosin, and examined.

Experimental Protocol. Prior to vaccination the mice were exposed continuously for 3 months to one of the following three environmental conditions:

- 2.0 ppm of $NO_2$ (2 ppm $NO_2$)
- 0.5 ppm of $NO_2$ with daily 1-hr peaks of 2.0 ppm of $NO_2$ for 5 days/week (0.5/2 ppm $NO_2$)
- filtered air (0 ppm $NO_2$)

After the 3-month exposure, the mice were vaccinated with influenza vaccine by a single subcutaneous injection in the dorsal thoracic area and thereafter held in either an $NO_2$ environment or filtered air. Groups of 14 to 20 mice were killed at 2,4,8,12,16,20,24, and 28 weeks after vaccination. Sera from two mice were pooled and assayed for HI and SN antibodies and for immunoglobulin levels. To determine the protective effect of the vaccine, as measured by mortality rates and lung lesion scores, groups of 20 mice were challenged with $A_2$/Taiwan influenza virus at 4, 16, and 28 weeks after the vaccination. Table I-1 outlines the protocol for the entire study.

Statistical Analysis. As appropriate, the experimental results were subjected to statistical analysis and the significance of the observed differences was reported at the $\leq$5% probability level. The immunoglobulin level data were analyzed by a two-way analysis of variance and a multiple range test was used to determine the ranking and significance of the observed differences. The significance of the differences in mortality rates was determined by the normal approximation with correction for continuity (K. S. Brownlee, Statistical Theory and Methodology in Science and Engineering, p. 121, 1960, John Wiley and Sons, New York). This method is equivalent to the chi-square test applied with a 2 x 2 contingency table, except for the use of corrections for continuity. The Student t-test was used for statistical analysis of the lung lesion scores.

Table I-1

PROTOCOL FOR IMMUNOLOGICAL STUDIES IN MICE
EXPOSED TO NITROGEN DIOXIDE

| Pre-Vaccination $NO_2$ Exposure | Vaccination Treatment | Post-Vaccination $NO_2$ Exposure for 2,4,8,12,16,20,24,28 wks | | Infectious Challenge at 4,16,28 wks |
|---|---|---|---|---|
| A. 2 ppm | (1) Vaccine (n=440) | (1a) | 2 ppm Ab & Ag at each period (n=160) | At each period (n=60) |
| | | (1b) | 0 ppm Ab & Ig at each period (n=160) | At each period (n=60) |
| | (2) Saline (n=120) | (2a) | 2 ppm (n=60) | At each period (n=60) |
| | | (2b) | 0 ppm (n=60) | At each period (n=60) |
| B. 0.5 ppm with daily 1 hr peaks of 2 ppm | Identical experimental groups and observations as the above but exposed to 0.5 ppm $NO_2$ with daily 1 hr peaks of 2.0 ppm $NO_2$ | | | |
| C. 0 ppm | (1) Vaccine (n=660) | (1a) | 0 ppm Ab & Ig at each period (n=160) | At each period (n=60) |
| | | (1b) | 0.5/2 ppm Ab & Ig at each period (n=160) | At each period (n=60) |
| | | (1c) | 2 ppm Ab & Ig at each period (n=160) | At each period (n=60) |
| | (2) Saline | (2a) | 0 ppm (n=60) | At each period (n=60) |
| | | (2b) | 0.5/2 ppm (n=60) | At each period (n=60) |
| | | (2c) | 2 ppm (n=60) | At each period (n=60) |

Sera for initial HI and SN antibody titers were obtained from groups of 20 mice after the 3 months pre-exposure to each experimental condition.

Ab and IG Studies: HI and SN antibody and immunoglobulin assays on pooled serum samples consisting of 7-10 groups of 2 mice.

Mortality Studies: Infectious challenge with airborne A2/Taiwan influenza virus of 3 groups of 20 mice.

## C. Results

### 1. Body Weights

Mice from two randomly selected cages in each of the three exposure groups were weighed at weekly intervals. The initial mean body weights of 22 mice in 2.0 ppm $NO_2$ chamber, 21 mice in 0.5/2 ppm $NO_2$ chamber, and 30 control mice were 30.0, 30.0, and 32.5 g. At the time of vaccination, i.e. 12 weeks after initiation of the exposures, mice exposed to $NO_2$ had gained an average of 13 g while the control mice gained 9.5 g. All three groups of mice weighed 44.5 g at 21 weeks after initiation of the exposure and after 28 weeks the mean weights were 44.2, 45.9 and 45.1 g for the 2.0 ppm $NO_2$ and 0.5/2 ppm $NO_2$, and control mice, respectively. The respective mean weights at 40 weeks were 45.4, 46.9 and 46.3 g. Thus, all groups gained weight consistent throughout the study.

### 2. Serology

The differences in HI antibody levels between experimental and control mice observed throughout the study were not significant (fourfold) and the HI antibody titers appeared to decline at the same rate in all animals (Table I-2). Some differences were noted in the levels of SN antibody. Two weeks after vaccination the SN antibody titers appeared depressed in mice exposed to $NO_2$ (Table 2). A significant fourfold difference in SN antibody titers was seen between control mice (1:34) and mice continuously exposed to 0.5/2 ppm of $NO_2$, and those held in filtered air before vaccination and 2 or 0.5/2 ppm of $NO_2$ after vaccination (<1:8 to 1:8). At the 4- and 8-week periods, the SN titers were comparable for all groups. The decline in SN titers in all groups began approximately 12 weeks after vaccination and appeared unaffected by the experimental treatment.

Table I-2

MEAN HI AND SN RECIPROCAL TITERS IN VACCINATED MICE EXPOSED TO NITROGEN DIOXIDE[a]

| $NO_2$, ppm | | HI Titer[b] | | | | | | | | SN Titer | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 Months Pre-Vacc. | Post-Vacc. | Weeks after Vaccination | | | | | | | | Weeks after Vaccination | | | | | | | |
| | | 2 | 4 | 8 | 12 | 16 | 20 | 24 | 28 | 2 | 4 | 8 | 12 | 16 | 20 | 24 | 28 |
| 0 | 0 | 18 | 16 | 14 | 14 | 20 | 7 | <8 | 9 | 34 | 23 | 23 | 10 | 21 | 7 | 10 | 17 |
| 0.5/2 | 0.5/2 | 13 | 16 | 10 | 12 | 23 | 14 | <8 | <8 | <8 | 31 | 29 | 7 | 17 | 8 | 9 | 9 |
| 2 | 2 | 17 | 11 | 7 | 7 | 19 | 14 | <8 | <8 | 12 | 46 | 26 | 14 | 12 | 7 | 11 | 8 |
| 0 | 0.5/2 | 33 | 16 | 11 | 30 | 33 | 14 | <8 | c | 8 | 26 | 19 | 15 | 21 | 10 | 8 | c |
| 0 | 2 | 12 | 8 | 8 | 14 | 13 | 10 | <8 | 8 | 8 | 26 | 36 | 11 | 8 | 6 | 17 | 12 |
| 0.5/2 | 0 | 9 | 19 | 16 | 14 | 13 | 7 | <8 | <8 | 10 | 15 | 21 | 8 | 25 | 12 | 17 | 16 |
| 2 | 0 | 6 | 23 | 12 | 15 | 14 | 16 | <8 | <8 | 20 | 40 | 21 | 17 | 17 | 24 | 16 | 11 |

[a] Each titer represents a mean of seven to ten pools of serum, with two mice used per pool.

[b] Four antigen units of egg-grown $A_2$/Taiwan influenza virus were used.

2 week serum samples tested against 258-30 EID50 units
4,8,24,28 week serum samples tested against 21-47 EID50 units
12 and 16 week serum samples tested against 100-210 EID50 units
20 week serum samples tested against 47-320 EID50 units

[c] Animals not available for bleeding.

The rates of HI and SN seroconversion (expressed as percentage of mice showing titers $\geq 8$) in vaccinated mice held in various environments are shown in Table I-3. Two weeks after vaccination the HI seroconversion rate was similar in all groups except those exposed to 0.5/2 or 2 ppm of $NO_2$ before vaccination and maintained in filtered air after vaccination. The seroconversion rate among those mice was markedly reduced (20 and 29%) relative to that in the remaining groups of mice (50 to 100%). In those mice exposed to 2 ppm $NO_2$ before and after vaccination, HI seroconversion rate decreased from 4 until 2 weeks after vaccination; thereafter, no distinct pattern of differences in HI seroconversion rate could be found.

At 2 weeks after vaccination the SN seroconversion rate in the control mice was 100% whereas none of the mice exposed to 0.5/2 ppm of $NO_2$ seroconverted. In the same period, the seroconversion rates for the remaining experimental groups of mice were also depressed, ranging from 40 to 57%. At 4 weeks after vaccination, the seroconversion rate ranged from 80 to 100% in the various experimental groups. The seroconversion rate appeared to decrease at 12 weeks after vaccination in a manner similar to the decline in SN antibody levels and HI seroconversion rates. In general, the HI seroconversion rate decreased more rapidly than the SN rate.

3. Immunoglobulins

Immunoglobulin levels of nonvaccinated mice exposed to $NO_2$ for 3 months differed significantly from those of mice held in filtered air (Table I-4). Mice exposed to either 2 or 0.5/2 ppm $NO_2$ showed a significant decrease in serum IgA and a significant increase in $IgG_1$ levels relative to control mice exposed to filtered air. The concentrations of IgM and $IgG_2$ were higher for all mice held in the two $NO_2$ environments, although the statistical significance could be ascertained only for the mice exposed to 0.5/2 ppm $NO_2$.

Table I-3

RATE OF SEROCONVERSION IN VACCINATED MICE[a]

| $NO_2$, ppm | | HI Response, % | | | | | | | | SN Response, % | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Weeks after Vaccination | | | | | | | | Weeks after Vaccination | | | | | | | |
| 3 Months Pre-Vacc. | Post-Vacc. | 2 | 4 | 8 | 12 | 16 | 20 | 24 | 28 | 2 | 4 | 8 | 12 | 16 | 20 | 24 | 28 |
| 0 | 0 | 71 | 75 | 71 | 57 | 71 | 29 | 0 | 25 | 100 | 100 | 100 | 57 | 57 | 33 | 43 | 62 |
| 0.5/2 | 0.5/2 | 60 | 67 | 29 | 57 | 86 | 43 | 0 | 0 | 0 | 100 | 71 | 43 | 67 | 43 | 67 | 50 |
| 2 | 2 | 70 | 25 | 29 | 14 | 57 | 57 | 0 | 0 | 50 | 88 | 86 | 33 | 71 | 43 | 33 | 67 |
| 0 | 0.5/2 | 100 | 71 | 67 | 83 | 67 | 60 | 0 | b | 43 | 86 | 67 | 67 | 83 | 60 | 40 | b |
| 0 | 2 | 50 | 30 | 33 | 43 | 57 | 57 | 0 | 20 | 40 | 80 | 67 | 43 | 43 | 29 | 71 | 40 |
| 0.5/2 | 0 | 20 | 60 | 67 | 56 | 29 | 14 | 14 | 14 | 56 | 80 | 63 | 44 | 43 | 57 | 57 | 57 |
| 2 | 0 | 29 | 86 | 44 | 44 | 43 | 57 | 14 | 16 | 57 | 86 | 78 | 56 | 43 | 57 | 100 | 50 |

[a] Each point represents a mean of seven to ten pools of serum, with two mice used per pool.

[b] Animals not available for bleeding.

Table I-4

SERUM IMMUNOGLOBULIN CONCENTRATIONS IN MICE EXPOSED TO NITROGEN DIOXIDE FOR THREE MONTHS

| $NO_2$, ppm | Immunoglobulin, mg/ml | | | |
|---|---|---|---|---|
| | IgA | IgM | IgG1 | IgG2 |
| 0 | 0.71 | 0.215 | 2.65 | 4.30 |
| 0.5/2 | 0.56↓ | 0.275↑ | 5.00↑ | 5.30↑ |
| 2 | 0.46↓ | 0.225 | 3.70↑ | 4.72 |

Arrows indicate either a significant increase or decrease in the serum immunoglobulin concentration relative to filtered-air values.

Table I-5 shows the concentrations of immunoglobulins in sera of vaccinated mice exposed to $NO_2$ for various periods. Assessing the influence of $NO_2$ on immunoglobulin concentrations over the 28-week exposure period was difficult, inasmuch as age markedly affects the levels of the various immunoglobulins (Bosma, et al. J. Immunol. 99, 420, 1967; Rector and Carter, J. Immunol. 110, 1591, 1973). To eliminate the age bias, a two-way analysis of variance was used whereby the age factor was removed as a source of variation. Thereafter, a multiple-range test was applied to the data to establish the ranking and significance of the differences in mean immunoglobulin concentrations between the various experimental groups. As shown in Table I-6, mean concentrations with common superscript did not differ significantly within each immunoglobulin.

Table I-5

SERUM IMMUNOGLOBULIN CONCENTRATIONS IN VACCINATED MICE EXPOSED TO VARIOUS NITROGEN DIOXIDE CONDITIONS

| $NO_2$, ppm | | | Immunoglobulin, mg/ml | | | |
|---|---|---|---|---|---|---|
| 3 Months Pre-Vacc. | Post-Vacc. | Weeks Post-Vacc. | IgA | IgM | IgG1 | IgG2 |
| 0 | 0 | 2 | 0.700 | 0.200 | 1.75 | 4.30 |
| 0.5/2 | 0.5/2 | | 0.620 | 0.235 | 5.50↑ | 5.20 |
| 2 | 2 | | 0.435↓ | 0.260 | 3.80 | 4.35 |
| 0 | 0.5/2 | | 0.500↓ | 0.115↓ | 3.95 | 4.30 |
| 0 | 2 | | 0.535 | 0.190 | 5.60↑ | 5.50↑ |
| 0.5/2 | 0 | | 0.600 | 0.250 | 2.15 | 4.10 |
| 2 | 0 | | 0.550 | 0.260 | 2.70 | 3.80 |
| 0 | 0 | 4 | 0.515 | 0.180 | 3.75 | 4.05 |
| 0.5/2 | 0.5/2 | | 0.560 | 0.245↑ | 5.40 | 6.50↑ |
| 2 | 2 | | 0.700 | 0.220 | 4.40 | 5.10 |
| 0 | 0.5/2 | | 0.770↑ | 0.190 | 5.85↑ | 6.55↑ |
| 0 | 2 | | 0.740 | 0.260 | 6.80↑ | 6.30↑ |
| 0.5/2 | 0 | | 0.380 | 0.200 | 3.75 | 4.75 |
| 2 | 0 | | 0.460 | 0.180 | 5.00 | 5.22 |
| 0 | 0 | 8 | 0.830 | 0.215 | 5.00 | 5.60 |
| 0.5/2 | 0.5/2 | | 0.740 | 0.235 | 7.40↑ | 6.85 |
| 2 | 2 | | 0.510↓ | 0.130↓ | 6.20 | 5.22 |
| 0 | 0.5/2 | | 1.200↑ | 0.175 | 5.60 | 6.30 |
| 0 | 2 | | 0.630 | 0.210 | 4.50 | 5.85 |
| 0.5/2 | 0 | | 0.800 | 0.225 | 6.30 | 6.00 |
| 2 | 0 | | 0.650↓ | 0.220 | 6.20 | 6.75 |
| 0 | 0 | 12 | 1.046 | 0.182 | 4.80 | 4.50 |
| 0.5/2 | 0.5/2 | | 0.824 | 0.211 | 9.20↑ | 5.59 |
| 2 | 2 | | 1.052 | 0.196 | 5.37 | 6.40↑ |
| 0 | 0.5/2 | | 1.117 | 0.246 | 6.20 | 6.19↑ |
| 0 | 2 | | 1.297 | 0.259↑ | 6.30 | 7.84↑ |
| 0.5/2 | 0 | | 0.840 | 0.166 | 4.28 | 4.45 |
| 2 | 0 | | 0.543↓ | 0.200 | 7.20 | 5.38 |
| 0 | 0 | 16 | 0.973 | 0.160 | 7.50 | 5.29 |
| 0.5/2 | 0.5/2 | | 1.088 | 0.254 | 5.53 | 6.08 |
| 2 | 2 | | 1.578↑ | 0.231 | 6.60 | 7.61↑ |
| 0 | 0.5/2 | | 1.881↑ | 0.244 | 6.75 | 6.22 |
| 0 | 2 | | 1.909↑ | 0.232 | 8.10 | 7.35↑ |
| 0.5/2 | 0 | | 0.856 | 0.213 | 8.60 | 6.78 |
| 2 | 0 | | 1.297 | 0.235 | 8.25 | 7.35 |

Table I-5 (continued)

| $NO_2$, ppm | | Weeks | Immunoglobulin, mg/ml | | | |
|---|---|---|---|---|---|---|
| 3 Months Pre-Vacc. | Post-Vacc. | Post-Vacc. | IgA | IgM | $IgG_1$ | $IgG_2$ |
| 0 | 0 | 20 | 1.080 | 0.254 | 4.60 | 6.70 |
| 0.5/2 | 0.5/2 | | 1.920↑ | 0.227 | 5.57 | 8.08 |
| 2 | 2 | | 1.690 | 0.274 | 6.10 | 6.15 |
| 0 | 0.5/2 | | 1.650 | 0.305 | 6.40 | 7.22 |
| 0 | 2 | | 1.430 | 0.165 | 7.25↑ | 5.88 |
| 0.5/2 | 0 | | 1.000 | 0.312 | 5.80 | 8.22 |
| 2 | 0 | | 1.250 | 0.300 | 7.80↑ | 8.94 |
| 0 | 0 | 24 | 1.720 | 0.233 | 6.70 | 6.62 |
| 0.5/2 | 0.5/2 | | 1.240 | 0.310 | 6.50 | 7.10 |
| 2 | 2 | | 1.680 | 0.187 | 3.44 | 4.11↓ |
| 0 | 0.5/2 | | 1.620 | 0.237 | 4.44 | 6.19 |
| 0 | 2 | | 1.120 | 0.241 | 5.85 | 6.62 |
| 0.5/2 | 0 | | 1.460 | 0.270 | 7.60 | 9.22 |
| 2 | 0 | | 1.770 | 0.300 | 8.70 | 9.49↑ |
| 0 | 0 | 28 | 0.23 | 0.241 | 8.75 | 7.31 |
| 0.5/2 | 0.5/2 | | 1.19 | 0.250 | 7.60 | 5.98 |
| 2 | 2 | | 2.37↑ | 0.252 | 6.80 | 5.56 |
| 0 | 0.5/2 | | NO ANIMALS AVAILABLE | | | |
| 0 | 2 | | 2.52↑ | 0.384↑ | 8.70 | 6.86 |
| 0.5/2 | 0 | | 1.28 | 3.379↑ | 7.60 | 7.14 |
| 2 | 0 | | 1.53 | 0.497↑ | 4.84↓ | 5.69 |

Arrows indicate either a significant increase or decrease in the serum immunoglobulin concentration relative to that of filtered-air controls bled at same time.

Table I-6

EFFECT OF EXPOSURE TO NITROGEN DIOXIDE ON IMMUNOGLOBULINS CONCENTRATION IN SERA OF VACCINATED MICE

| $NO_2$, ppm | | Number | | Immunoglobulin, mg/ml | |
|---|---|---|---|---|---|
| 3 Months Pre-Vacc. | Post-Vacc. | of Assays | IG | Mean | 95% C.L. |
| 0.5/2 | 0 | 68 | IgA | 0.829[a] | 0.755-0.906 |
| 2 | 0 | 59 | | 0.861[a] | 0.784-0.954 |
| 0.5/2 | 0.5/2 | 54 | | 0.918[a] | 0.829-1.018 |
| 0 | 0 | 60 | | 0.936[ab] | 0.851-1.032 |
| 2 | 2 | 56 | | 1.032[bc] | 0.936-1.147 |
| 0 | 2 | 61 | | 1.073[bc] | 0.973-1.187 |
| 0 | 0.5/2 | 42 | | 1.163[c] | 1.032-1.315 |
| 0 | 0 | 61 | IgM | 0.207[a] | 0.188-0.227 |
| 2 | 2 | 56 | | 0.217[ab] | 0.196-0.237 |
| 0 | 0.5/2 | 42 | | 0.217[ab] | 0.194-0.244 |
| 0 | 2 | 61 | | 0.237[bc] | 0.217-0.259 |
| 0.5/2 | 0.5/2 | 53 | | 0.244[bc] | 0.219-0.267 |
| 0.5/2 | 0 | 69 | | 0.246[bc] | 0.225-0.267 |
| 2 | 0 | 59 | | 0.263[c] | 0.241-0.288 |
| 0 | 0 | 60 | $IgG_1$ | 5.02[a] | 4.37-5.74 |
| 2 | 2 | 56 | | 5.17[ab] | 4.47-6.02 |
| 0.5/2 | - | 69 | | 5.37[ab] | 4.70-6.11 |
| 0 | 0.5/2 | 42 | | 5.41[ab] | 4.57-6.46 |
| 2 | 0 | 59 | | 6.21[bc] | 5.37-7.23 |
| 0.5/2 | 0.5/2 | 54 | | 6.88[c] | 5.88-8.06 |
| 0 | 2 | 61 | | 6.94[c] | 5.97-8.13 |
| 0 | 0 | 60 | $IgG_2$ | 5.29[a] | 4.95-5.69 |
| 2 | 2 | 56 | | 5.32[a] | 4.95-5.72 |
| 0.5/2 | 0 | 68 | | 5.91[b] | 5.56-6.33 |
| 0 | 0.5/2 | 42 | | 5.98[b] | 5.50-6.51 |
| 2 | 0 | 59 | | 6.15[b] | 5.72-6.59 |
| 0.5/2 | 0.5/2 | 54 | | 6.26[b] | 5.85-6.78 |
| 0 | 2 | 61 | | 6.36[b] | 5.95-6.86 |

$NO_2$ Exposure groups are listed in the order of increasing immunoglobulin concentration.

Means with a common superscript, within each immunoglobulin class, are not significantly different at $P \leq 5\%$.

Exposure to $NO_2$ did not influence the concentration of IgA. The exception was the group of mice maintained in filtered air before the vaccination and exposed to the 0.5/2 ppm $NO_2$ atmosphere after the vaccination, which showed a significant increase in IgA concentration.

The IgM data indicated that concentrations of this immunoglobulin in all groups of mice exposed to $NO_2$ were higher than that in control mice maintained in filtered air. Statistically significant increases in IgM levels over the control groups were observed in groups of mice subjected to: 0-2 ppm $NO_2$, 0.5/2-0.5/2 ppm $NO_2$, 0.5/2-0 ppm $NO_2$ and 2-0 ppm $NO_2$. Moreover, IgM concentrations in the groups of mice exposed to 2 ppm for 3 months before vaccination then maintained in filtered air was significantly higher than in the 2-2 ppm $NO_2$ and 0-0.5/2 ppm $NO_2$ exposure groups.

The $IgG_1$ and $IgG_2$ levels showed a similar pattern in that the control group had the lowest mean concentration over the 28-week period. The three groups that showed significantly higher $IgG_1$ concentrations than the controls were 2-0 ppm $NO_2$, 0.5-2-0.5/2 ppm $NO_2$, and 0-2 ppm $NO_2$. In all but the group of mice exposed continuously to 2.0 ppm $NO_2$, $IgG_2$ concentrations increased significantly. The 0.5/2-0.5/2 ppm $NO_2$, 0-2 ppm $NO_2$, and 2-0 ppm $NO_2$ exposure groups showed a significant elevation in this immunoglobulin subclass as well.

It is interesting that continuous exposure to 2 ppm $NO_2$ before and after vaccination did not significantly alter the levels of any immunoglobulin class. However, exposure of 0-2 ppm $NO_2$, 2-0 ppm $NO_2$, and 0.5/2-0.5/2 ppm $NO_2$ resulted in significantly elevated IgM, $IgG_1$ and $IgG_2$ levels. The common factor shared by these three groups was the change from one environment to another.

Studies of immunoglobulin response to influenza infection or vaccination have concentrated on the humoral response of IgM and IgG, considered to be responsible for, respectively, complement fixation (CF) and hemagglutination titers (HI) (Daugharty, et al. J. Immunol. 109, 849, 1972). Increased levels of serum IgA have recently been identified in the monomeric form, after influenza infection or vaccination. Thus, all three immunoglobulins appear to play a significant role in the host's response to influenza infection or vaccination.

Our study showed that mice exposed to $NO_2$ before or after vaccination produced levels of IgM, $IgG_1$, $IgG_2$ higher than those in control animals. More specifically, mice consistently showing elevated levels of these immunoglobulins were those exposed to air for 3 months before vaccination and then to 2 ppm $NO_2$, those exposed to 2 ppm $NO_2$ for 3 months before vaccination then to filtered air, and those exposed continuously to 0.5 ppm $NO_2$ with daily peaks of 2 ppm $NO_2$ before and after vaccination. In each case the concentrations of $NO_2$ were varied, while exposure to fixed concentrations of $NO_2$ before and after vaccination, even at 2 ppm $NO_2$, did not result in elevation of any immunoglobulin above control levels. Increase in immunoglobulins can be postulated to affect a protective immune response adversely since high levels of circulating IgG have been shown to inhibit the production of specific antibody synthesis (R. Waldman, personal communication, 1973). Indeed, these three groups of mice, when challenged with live airborne influenza $A_2$/Taiwan virus, exhibited the highest mortality rate at 16 and 28 weeks after vaccination.

4. Infectious Challenge

Vaccinated mice and nonvaccinated mice injected with saline were challenged with airborne $A_2$/Taiwan influenza virus at 4, 16, and 28 weeks after vaccination. Comparisons between observed frequencies of mortality were made using the chi-square

test with a 2 x 2 contingency table, and corrections for continutiy. The Student t-test was used for statistical evaluation of the lung lesion data.

a. Four Weeks after Vaccination. The vaccine appeared to afford satisfactory protection against the infectious challenge in mice challenged with airborne influenza virus 4 weeks after vaccination (Table I-7). Because of the low challenge dose, only 15% mortality was observed in the nonvaccinated mice. Thus, the significance of the protection provided by vaccination could be ascertained only for

- mice continuously exposed to 0.5/2 ppm of $NO_2$,
- mice held in filtered air before vaccination then exposed to 2 ppm of $NO_2$, and
- mice exposed to 0.5/2 ppm $NO_2$ before vaccination then maintained in filtered air.

As Table I-8 shows, lung lesion scores confirm the mortality data. Although the differences in mortality rates within the vaccinated and nonvaccinated groups were not significant, lung lesions, which serve as a highly sensitive indicator of influenza infection, were significantly more severe in several groups of mice. Within the group of vaccinated mice challenged with the infectious virus, a significant increase in lung lesions was observed in mice exposed to 0.5/2 or 2 ppm of $NO_2$ before vaccination and to filtered air after the vaccination. Nevertheless, in all instances the vaccine provided sufficient protection so that, irrespective of the experimental exposure conditions, the extent of lung lesions was significantly lower in vaccinated than in nonvaccinated mice.

Table I-7

MORTALITY RATE IN MICE EXPOSED TO NITROGEN DIOXIDE AND CHALLENGED WITH INFLUENZA VIRUS 4, 16 AND 28 WEEKS AFTER VACCINATION

| $NO_2$, ppm | | 4 Weeks | | | | 16 Weeks | | | | 28 Weeks | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 Months Pre-Vacc. | Post-Vacc. | Saline | | Vaccine | | Saline | | Vaccine | | Saline | | Vaccine | |
| | | D/T | % | D/T | % | D/T | % | D/T | % | D/T | % | D/T | % |
| 0 | 0 | 3/20 | 15 | 0/20 | 0 | 14/15 | 93 | 1/15 | 7* | 25/26 | 96 | 9/25 | 26* |
| 0.5/2 | 0.5/2 | 9/20 | 45 | 0/20 | 0* | 13/15 | 87 | 4/15 | 27* | 18/20 | 90 | 6/12 | 50* |
| 2 | 2 | 1/19 | 5 | 0/20 | 0 | 13/15 | 87 | 1/15 | 7* | 11/12 | 92 | 7/15 | 47* |
| 0 | 0.5/2 | 3/20 | 15 | 0/19 | 0 | 10/15 | 67 | 3/15 | 20* | 5/5 | 100 | 2/10 | 20* |
| 0 | 2 | 7/20 | 35 | 0/18 | 0* | 15/15 | 100 | 4/15 | 27* | 13/15 | 87 | 9/15 | 60 |
| 0.5/2 | 0 | 9/20 | 45 | 2/20 | 10* | 13/15 | 87 | 1/15 | 7* | 17/23 | 74 | 8/25 | 32* |
| 2 | 0 | 7/20 | 35 | 3/19 | 16 | 11/15 | 73 | 6/15 | 40 | 12/14 | 86 | 10/15 | 67 |

* Significant differences between vaccinated and corresponding nonvaccinated mice.

Table I-8

LUNG LESIONS IN MICE EXPOSED TO NITROGEN DIOXIDE AND CHALLENGED WITH INFLUENZA VIRUS 4, 16 AND 28 WEEKS AFTER VACCINATION

| $NO_2$, ppm | | Lung Lesion Scores[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 4 Weeks | | 16 Weeks | | 28 Weeks | |
| 3 Months Pre-Vacc. | Post-Vacc. | Saline | Vaccine | Saline | Vaccine | Saline | Vaccine |
| 0 | 0 | 1.95 | 0.30* | 4.80 | 1.27* | 4.88 | 2.40* |
| 0.5/2 | 0.5/2 | 3.20+ | 0.35* | 4.60 | 2.27* | 4.65 | 2.92* |
| 2 | 2 | 2.37 | 0.35* | 4.60 | 1.60* | 4.83 | 3.00* |
| 0 | 0.5/2 | 2.05 | 0.42 | 4.27 | 1.92 | 5.00 | 1.90* |
| 0 | 2 | 3.00+ | 0.44* | 5.00 | 2.40+ | 4.60 | 3.47 |
| 0.5/2 | 0 | 3.40+ | 1.15+ | 4.53 | 1.40* | 4.30 | 2.60* |
| 2 | 0 | 2.90 | 1.21+ | 4.13 | 2.60+ | 4.71 | 3.80+ |

[a] Each score represents the mean lung lesion score of 14 to 20 mice.

* Significant differences between vaccinated and corresponding non-vaccinated mice.

\+ Significant differences within vaccinated or nonvaccinated groups when compared with mice held in filtered air.

b. Sixteen Weeks after Vaccination. At 16 weeks after vaccination, the vaccine exerted protection in all groups except mice exposed to 2.0 ppm $NO_2$ before vaccination then held in filtered air (Table I-7). All vaccinated mice challenged with the virus showed significantly less severe lung lesions than the corresponding non-vaccinated control mice (Table I-8). Within the vaccinated groups, mice held in filtered air before vaccination then exposed to 2 ppm $NO_2$, or those exposed to 2 ppm $NO_2$ before vaccination then maintained in filtered air for 16 weeks had more severe influenza lung lesions than the vaccinated mice held continuously in filtered air.

c. Twenty-eight Weeks after Vaccination. The vaccine was protective in all but two groups of mice challenged with virus 28 weeks after vaccination (Table I-7). The two groups were mice held in filtered air prior to vaccination then exposed to 2 ppm $NO_2$ and mice exposed to 2 ppm $NO_2$ before vaccination then maintained in filtered air for 28 weeks before the infectious challenge.

Vaccinated mice held in these two environmental conditions had lung lesion scores that were similar to the corresponding non-vaccinated mice (Table I-8), an indication of increased susceptibility to infection. Within the vaccinated groups, only mice held in 2 ppm $NO_2$ for 3 months before vaccination then maintained in filtered air for 28 weeks and challenged with the infectious virus had more severe lung lesions than vaccinated mice held continuously in filtered air. Thus, this group of mice, when challenged at 4, 16, or 28 weeks after vaccination, appeared to be more susceptible to infection as measured by mortality rates and lung lesion scores.

Mice surviving the virus challenge at 16 and 28 weeks after vaccination were exsanguinated and the sera were assayed for HI antibody to determine the anamnestic response. Vaccinated mice in both the control and experimental groups showed a

consistent and excellent anamnestic response to the influenza virus infection (Table I-9). The primary immune response is that shown by nonvaccinated mice injected saline then challenged with influenza virus.

5. Lung Edema

The extent of lung edema as measured by the wet-to-dry lung-weight ratios was examined at the various period. In general, the differences in mean lung edema ratios between mice exposed to filtered air and those exposed to $NO_2$ were not significant (Table I-10). There were some marked differences in edema ratios among the various groups of mice exposed to $NO_2$ but no pattern was evident. Thus, after approximately 10 months exposure to $NO_2$, no significant differences in the lung edema ratios were found between mice exposed to $NO_2$ and filtered air.

6. Histopathology

Histopathological examination of lungs from mice vaccinated against influenza two weeks after vaccination revealed a mild subacute to acute pneumonitis with slight to mild bronchiolar epithelial hyperplasia. Many had mild peribronchiolar lymphocytic accumulations reflecting a baseline chronic murine pneumonia (Figure 1). Mild peripheral emphysema was also present in some of the mice. The various $NO_2$ exposure conditions did not appear to influence the type or the extent of pathological changes.

The lungs of mice vaccinated against influenza examined four weeks after the vaccination showed similar pathology. A slight subacute interstial pneumonitis was present with baseline chronic murine pneumonia. These lungs were in a marked contrast to those of mice challenged with $A_2$/Taiwan influenza virus 2 weeks after the vaccination and killed 2 weeks later. A mild to moderate amount of focal parabronchial and peribronchiolar bronchoalveolar proliferation (pulmonary adenomatosis, alveolar fetalization, bronchiolar adenomatoid lesion) was present (Figure 2). The alveolar lining cells were hyperplastic and gave the appearance

Table I-9

MEAN HI TITERS IN SURVIVING MICE EXPOSED TO NITROGEN DIOXIDE AND INFLUENZA VIRUS AT 16 AND 28 WEEKS AFTER VACCINATION

| $NO_2$, ppm | | Reciprocal HI Titer | | | |
|---|---|---|---|---|---|
| | | 16 Weeks | | 28 Weeks | |
| 3 Months Pre-Vacc. | Post-Vacc. | Saline | Vaccine | Saline | Vaccine |
| 0 | 0 | 8 (1) | 243 (14) | 24 (1) | 151 (16) |
| 0.5/2 | 0.5/2 | 288 (2) | 277 (11) | 24 (2) | 99 (6) |
| 2 | 2 | 28 (2) | 294 (14) | 24 (1) | 155 (8) |
| 0 | 0.5/2 | 28 (5) | 309 (12) | (0) | 101 (8) |
| 0 | 2 | (0) | 256 (11) | <16 (2) | 99 (6) |
| 0.5/2 | 0 | N.D.* | 300 (11) | 136 (6) | 267 (17) |
| 2 | 0 | 36 (4) | 272 (9) | 24 (2) | 128 (5) |

Number of surviving mice shown in parentheses.

* Not determined.

Table I-10

WET:DRY LUNG RATIO IN MICE EXPOSED TO NITROGEN DIOXIDE

| $NO_2$, ppm | | Ratio, wet-to-dry lung weight Weeks after Vaccination: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 3 Months Pre-Vacc. | Post-Vacc. | 2 | 4 | 8 | 12 | 16 | 20 | 24 | 28 |
| 0 | 0 | 4.53 | 4.68 | 4.64 | 4.11 | 4.70 | 4.53 | 4.52 | 4.07 |
| 0.5/2 | 0.5/2 | 4.54 | 4.50 | 4.55 | 4.79* | 4.40 | 4.45 | 4.21 | 4.26 |
| 2 | 2 | 4.70 | 4.54 | 4.65 | 3.88 | 5.06 | 4.85 | 4.25 | 4.18 |
| 0 | 0.5/2 | 4.78 | 4.52 | 4.48 | 4.72 | 4.43 | 4.54 | 4.35 | (a) |
| 0 | 2 | 4.66 | 4.39 | 4.64 | 4.08 | 4.55 | 4.60 | 4.39 | 4.10 |
| 0.5/2 | 0 | 4.58 | 4.58 | 4.50 | 4.44 | 4.72 | 4.38 | 4.26 | 3.96 |
| 2 | 0 | 4.46 | 4.87 | 4.54 | 4.60 | 4.64 | 4.48 | 4.40 | 3.97 |

* Significant differences when compared with mice exposed to filtered air.

(a) Animals not available.

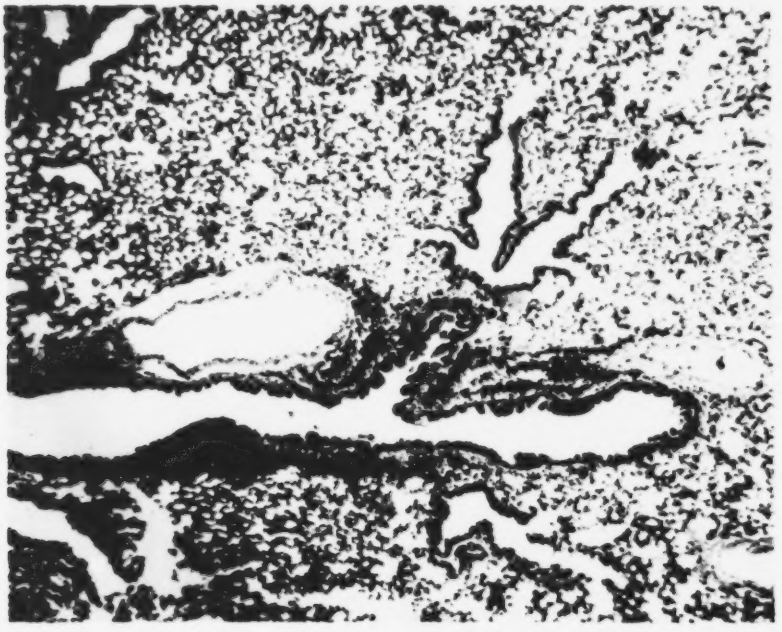

FIGURE 1: Mild parabronchial lymphocytic accumulations indicative of chronic murine pneumonia in lungs of vaccinated mouse exposed for 3-1/2 months to 0.5 ppm $NO_2$ with daily 1 hr peaks of 2 ppm $NO_2$. 64x

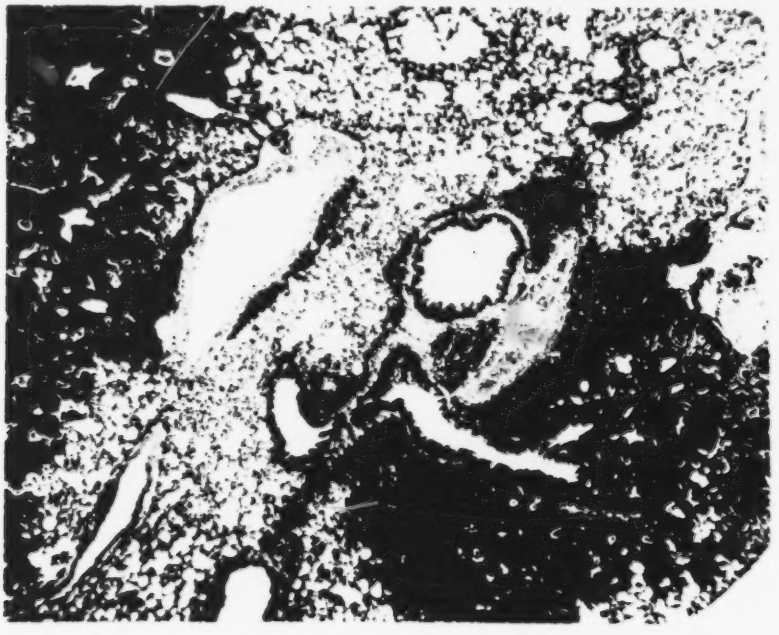

FIGURE 2: Moderate local peribronchial and parabronchial broncho-alveolar proliferation in lungs of vaccinated mouse challenged with live influenza virus. The mouse was exposed for 3 months to 0.5 ppm $NO_2$ with daily 1 hr peaks of 2 ppm $NO_2$ before vaccination and held for 1 month in filtered air after vaccination. 48x

of a cuboidal to columnar epithelium. Some of the cells possessed well-developed cilia. Cellular atypia was present in some of these proliferative lesions, but the extent was not sufficient for them to be considered anaplastic (Figure 3). Subacute interstitial pneumonitis, slight marginal emphysema, and slight chronic murine pneumonic changes were also present. The lungs of nonvaccinated mice challenged with virus and examined at 4 weeks, had the same type and extent of pathological changes. Differences in the extent or type of pathological changes observed in the lung tissues did not appear to be related to the $NO_2$ exposure conditions.

Lungs of vaccinated mice examined 16 and 28 weeks after vaccination had minimal changes, which were comparable to those observed in the lungs of vaccinated mice examined at 2 and 4 weeks. Alveologenic adenomas which are frequently found in older mice and are considered spontaneous tumors were present in two mice examined at 28 weeks.

Mild to severe focal peribronchial bronchoalveolar proliferative lesions were present in both vaccinated and nonvaccinated mice surviving the infectious virus challenge at 16 and 28 weeks after vaccination and killed two weeks later. Some areas of the proliferative reaction had moderate cellular atypia and were suggestive of carcinoma. A mild to marked peribronchial subacute inflammatory infiltrate, and moderate bronchial and bronchiolar epithelial hyperplasia (Figure 4) were present. No differences could be seen between nonvaccinated and vaccinated mice or the various $NO_2$ exposure groups.

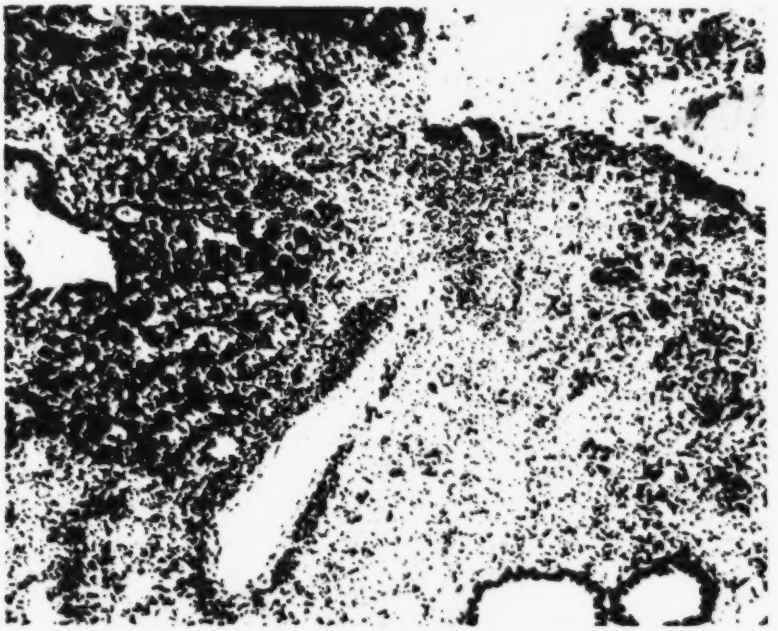

FIGURE 3: Proliferative lesions and cellular atypia suggestive of carcinoma in lung of vaccinated mouse exposed to 2 ppm $NO_2$ for 4 months and challenged with live influenza virus. 95x

FIGURE 4: Mild to marked peribronchial subacute inflammatory infiltrate and moderate bronchial and bronchiolar epithelial hyperplasia in lung of vaccinated mouse challenged with live influenza virus, exposed to filtered air. 122x

## D. Summary

Swiss albino mice were continuously exposed to either 2 ppm $NO_2$, 0.5 ppm $NO_2$ with daily 1-hr peaks of 2 ppm $NO_2$ 5 days a week, or filtered air. After a 3 month exposure the mice were vaccinated with $A_2$/Taiwan influenza vaccine and the exposures to the various environmental conditions continued for an additional 28 week period. At various time intervals sera were obtained for HI and SN antibody and immunoglobulin assays. In addition, at 4, 16 and 28 weeks after vaccination groups of mice were challenged by the respiratory route with airborne live $A_2$/Taiwan influenza virus. The mortality rates, lung lesion scores, and histopathological changes in lung tissue were observed.

Throughout the experiment all groups of mice gained weight consistently irrespective whether or not exposed to $NO_2$. Similarily, the formation and decline of HI antibody titers did not appear to be influenced by the environmental exposures. However, within 2 weeks after vaccination a significant depression of SN antibody titers was observed in mice continuously exposed to 0.5 ppm $NO_2$ with 2 ppm $NO_2$ peaks and those held in filtered air before vaccination and 2 ppm $NO_2$ or 0.5 ppm $NO_2$ with 2 ppm $NO_2$ peaks after the vaccination. Furthermore, at the same time period the SN seroconversion rate among mice continuously exposed to 0.5 ppm $NO_2$ with 2 ppm $NO_2$ peaks was 0% while in those exposed to filtered air it was 100%. After the initial 2 week period the SN titers did not differ significantly between the various experimental groups.

Immunoglobulin levels of mice exposed to $NO_2$ for 3 months prior to vaccination differed significantly from those held in filtered air. There was a significant decrease in serum IgA and a significant increase in $IgG_1$, $IgG_2$, and IgM levels. During the 28 week period following vaccination, exposure to $NO_2$ did not influence the concentration of IgA. However, IgM, $IgG_1$ and $IgG_2$ levels in all mice exposed to $NO_2$ were higher than in mice maintained in filtered air. More specifically, mice consistently showing statistically higher immunoglobulin levels were those

- exposed to filtered air for 3 months then to 2 ppm $NO_2$,
- exposed to 2 ppm $NO_2$ for 3 months then to filtered air, and
- exposed continuously to 0.5 ppm $NO_2$ with daily peaks of 2 ppm $NO_2$.

These three groups of mice also exhibited the highest mortality rates and severest pulmonary lesions when challenged with live airborne $A_2$/Taiwan influenza virus at 16 and 28 weeks after vaccination.

Lung tissues of mice challenged with the live influenza virus and sacrificed 2 weeks later showed moderate to severe bronchoalveolar proliferative reaction. Small to large focal areas of parabronchial or peribronchiolar bronchoalveolar proliferation were present with ciliation and slight cellular atypia in some portions. Subacute interstitial pneumonitis, slight marginal emphysema, and chronic murine pneumonia were also present. The bronchoalveolar proliferation was absent from the lungs of vaccinated animals not subsequently challenged with virus. Variation of $NO_2$ treatment did not appear to influence the extent of the proliferative inflammatory reaction, nor lung edema.

Results of these studies suggest that fluctuations in environmental conditions represent a more significant factor in immune response than exposure to a single stressful atmosphere. Continuous exposure of mice for approximately 10 months to 2 ppm $NO_2$ did not appear to influence formation of antibodies or levels of immunoglobulins, nor their resistance to subsequent respiratory challenge with live influenza virus. Conversely, continuous exposure to 0.5 ppm $NO_2$ with daily 1-hr peaks of 2 ppm $NO_2$ appeared to depress the ability to form SN antibodies and significantly altered the levels of IgM, $IgG_1$ and $IgG_2$ immunoglobulins. Furthermore, these mice developed a more severe infection as reflected by increased mortality rates upon challenge with live influenza virus.

## II. IMMUNE RESPONSE IN MICE DURING A 4-WEEK EXPOSURE TO $NO_2$

### A. Introduction

This study was conducted to examine the immune response of vaccinated mice during a one month exposure to low levels of nitrogen dioxide ($NO_2$). Mice were held in ambient air for 6 weeks, vaccinated with purified influenza vaccine and then exposed to $NO_2$. Parameters measured were hemagglutination-inhibition (HI) and serum neutralizing (SN) antibody formation, serum immunoglobulin levels, and mortality rates in mice challenged with live influenza virus.

### B. Materials and Methods

Animals. Six-week-old specific-pathogen-free male Swiss albino mice, CD-1 strain, were obtained from Charles River Laboratories. After a six-week quarantine, the 3 month old mice were placed in the environmental chambers. During the exposures, the mice were removed from the chamber for one hour three times a week for maintenance. Clean cages were provided once a week and food and water were provided ad libitum.

Influenza Virus. Mouse-adapted influenza $A_2$/Taiwan/1/64 virus was passaged several times in mice and 20% lung suspension of the virus was used for all infectious challenges. Prior to use, the virus was identified by use of specific antiserum obtained from the National Institutes of Health.

Vaccine. Chick embryo $A_2$/Taiwan/1/64 influenza vaccine (Zonomune), Lot No. BP0549, was supplied by Eli Lilly and Company, Indianapolis. Mice were vaccinated by a single subcutaneous injection of approximately 279 CAA units in 0.1 ml vaccine.

HI and SN Titrations. Tests were performed in duplicate, by the microtiter method in disposable V-plates (Cooke Engineering Co., Alexandria, Va.) described by Davenport and Minuse (Diagnostic Procedures for Viral and Rickettsial Diseases, 3rd ed. Am. Public Health Assoc., New York, 1964). In all tests, 1% chicken red blood cells were used.

Four hemagglutinating units of antigen were used for the HI test. All antisera were heat-inactivated at 56°C for 30 min and treated with trypsin-periodate to remove nonspecific inhibitors of hemagglutination.

The protocol used for the SN test was similar to that described in the USPHS Requirements (Division of Biologics Standards, 6th rev., 1947). Sera were heat-inactivated at 56°C for 30 min, serially diluted, and incubated with an equal volume of influenza virus for 1 hr at 4°C. The serum-virus mixture was then tested in 10-day-old embryonated chicken eggs. The eggs were inoculated by the allantoic route with 0.1 ml of the virus-serum mixture, incubated at 37°C and harvested when an $EID_{50}$ (50% egg infectious dose) dose of 32 to 320 was attained as indicated by hemagglutination of the virus control. The $EID_{50}$ dose was determined by parallel infectivity tests in eggs by using a 0.1-ml virus-saline mixture. Phosphate buffered saline (PBS), PBS plus normal mouse serum, and normal mouse serum plus virus were inoculated into eggs as controls.

Immunoglobulin Concentration. Quantitative radial immunodiffusion plates for mouse immunoglobulins IgA, $IgG_1$, $IgG_2$, and IgM were procured from Meloy Laboratories, Inc., Springfield, Virginia. Reference standards obtained from pooled sera of normal mice were assayed daily, in duplicate, to provide quality control. Duplicate serum samples were placed in preformed wells, the plates incubated at 22°C for 18 hr and the radial diffusion diameters measured using a Bausch & Lomb 7x lens. Duplicate radial diffusion diameters were recorded for each of the pooled serum sample; consisting of seven to 10 pooled samples at each experimental point.

Nitrogen Dioxide Exposure. To maintain the control and experimental mice under similar conditions, three identical aluminum-lined chambers (4 x 6 x 6.5 ft) were used for the exposures. Randomly selected mice were housed in suspended wire cages, which were periodically rotated to various position on the cage racks. This assured a thorough and unbiased exposure to the experimental environment. A minute amount of $NO_2$ was continuously passed from a cylinder through a stainless steel tube into a glass mixing vessel where it was diluted and mixed with charcoal-filtered ambient air. The mixture was then passed into the $NO_2$ exposure chambers at a rate of 20 changes per hour. The same air flow pattern was maintained in the control chamber where the charcoal-filtered ambient air was used. To verify the homogeneity of $NO_2$, air samples were taken from different sections of the chambers and the $NO_2$ concentration determined and calculated by the Saltzman method. A Mast gas analyzer was used for continuous $NO_2$ monitoring. The mean temperature in the chambers was 24° $\pm$ 1°C.

Scoring Pulmonary Lesions. The extent of pulmonary lesions was expressed as the percentage of the total lung consolidated. A score of 1 represented 25% lung consolidation, 2 = 50%, 3 = 75%, 4 = 100%, and a score of 5 represented animals that died during the experiment.

Aerosol Challenge. Infectious respiratory challenge was conducted in a 350-liter plastic aerosol chamber (60 x 60 x 95 cm) installed within a microbiological safety cabinet. A University of Chicago Toxicity Laboratory type atomizer was used to produce a viral aerosol with a majority of <5$\mu$-mass-median-diameter particles. The microbial suspensions were fed to the atomizer by a 50-ml syringe activated by a motor-driven piston delivering the suspension at a rate of 0.4 ml/min. Filtered air was supplied to primary and secondary inlets of the atomizer at a flow rate of 33 liters/min. The chamber was maintained at 78 $\pm$ 6% RH and 24 $\pm$ 1°C.

The aerosol was sampled with an all-glass impinger (AGI-30) containing PBS with 0.2% bovine serum albumin as a collecting fluid. The inhaled dose was estimated on the basis of the concentration of the virus per liter of air, respiratory minute volume of the mice, and the duration of exposure to the aerosol.

For the infectious challenge, groups of mice were placed in the chamber and exposed to the aerosol for 5 to 6 min. After the challenge, the mice were air-washed for 10 min, removed from the aerosol chamber and held for 14 days in filter-capped cages in a clean-air, isolated animal room.

Experimental Protocol. After vaccination the mice were exposed continuously to one of the following three environmental conditions:

- 2.0 ppm of $NO_2$ (2 ppm $NO_2$)
- 0.5 ppm of $NO_2$ with daily 1-hr peaks of 2.0 ppm of $NO_2$ for 5 days/week (0.5/2 ppm $NO_2$)
- filtered air (0 ppm $NO_2$)

Groups of 14 to 20 mice were killed at 1, 2, and 4 weeks after vaccination. Sera from two mice were pooled and assayed for HI and SN antibodies and for immunoglobulin levels. To determine the protective effect of the vaccine, as measured by mortality rates and lung lesion scores, groups of 20 mice were challenged with $A_2$/Taiwan influenza virus at 1, 2, and 4 weeks after the vaccination.

Statistical Analysis. As appropriate, the experimental results were subjected to statistical analysis and the significance of the observed differences was reported at the $\leq$5% probability level. The immunoglobulin level data were analyzed by a two-way analysis of variance and a multiple range test was used to determine the ranking and significance of the observed differences. The significance of the differences in mortality rates was determined

by the normal approximation with correction for continuity (K. S. Brownlee, Statistical Theory and Methodology in Science and Engineering, p. 121, 1960, John Wiley and Sons, New York). This method is equivalent to the chi-square test applied with a 2 x 2 contingency table, except for the use of corrections for continuity. The Student t-test was used for statistical analysis of the lung lesion scores.

## C. Results

Antibody Formation. The HI and SN antibody responses were noted in all mice within one week after vaccination, but no significant, 4-fold, differences in the titers were observed throughout the 4 week exposure period (Table II-1). Similarly there were no significant differences in seroconversion rates, although some depression of seroconversion was observed in mice held in $NO_2$ atmospheres for 1 week. With some discrepancy, the overall pattern of antibody response was similar to that observed during the first month of the previously reported long-term chronic exposure to $NO_2$. The inconsistency of the observations could be in part ascribed to the vaccine per se. The vaccine used in this experiment was obtained at a different time. Although the label indicated that the vaccine was from the same lot as the one used during the long-term chronic exposure studies, nevertheless it appeared to be markedly more immunogenic. An additional factor contributing to the discrepancy was the pre-vaccination exposure. In the long-term chronic studies the mice were exposed to 2 ppm $NO_2$, 0.5/2 ppm $NO_2$ or filtered air for 3 months before vaccination. In this study all mice were exposed to filtered air for 1 1/2 months before the vaccination.

Table II-1

MEAN HI AND SN RECIPROCAL TITERS AND SEROCONVERSION RATES IN VACCINATED MICE EXPOSED TO $NO_2$

| $NO_2$, ppm | Antibody Titer | | | | | | Seroconversion, % | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HI[a] | | | SN[b] | | | HI | | | SN | | |
| | 1[c] | 2 | 4 | 1 | 2 | 4 | 1 | 2 | 4 | 1 | 2 | 4 |
| 0 | 37 | 30 | 54 | 34 | 19 | 103 | 90 | 100 | 100 | 100 | 78 | 100 |
| 0.5/2 | 27 | 46 | 56 | 23 | 71 | 156 | 70 | 100 | 100 | 86 | 100 | 100 |
| 2 | 26 | 40 | 105 | 19 | 33 | 109 | 60 | 100 | 100 | 80 | 100 | 100 |

[a] Four antigen units of egg grown $A_2$/Taiwan influenza virus used in the HI test.

[b] Tested against 23-100 $EID_{50}$ units.

[c] Weeks of exposure to $NO_2$ and after vaccination.



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Immunoglobulins. As shown in Table II-2 several changes were observed in serum immunoglobulin concentrations of vaccinated mice exposed to $NO_2$. During the 4 week period, IgA levels of vaccinated mice exposed to filtered air remained constant and did not differ markedly from the pre-vaccination level. Among mice exposed to 2 ppm $NO_2$ the IgA levels did not differ from pre-vaccination levels after 1 week, showed a marginal increase after 2 weeks and a significant increase (113%) after 4 weeks of exposure to $NO_2$. A similar response was observed in mice exposed to 0.5 ppm $NO_2$ with daily peaks of 2 ppm $NO_2$, where elevated IgA levels appeared already after 1 week of exposure, and persisted at 2 and 4 weeks.

All mice showed a rise in IgM concentration within 1 week after vaccination, and the levels remained elevated throughout the 4 week period, irrespective of the exposure condition. However, the pre-vaccination level of this immunoglobulin appeared to be lower than expected.

Immunoglobulin $IgG_1$, the subclass of IgG which is reported to appear first after immunization or infection (Ovary, Benacerraf, and Block, J. Exp. Med. 117, 951, 1963; Bloch, Kourelsky, Ovary, and Benacerraf, J. Exp. Med. 117, 965, 1963), increased significantly only in mice held in filtered air for 1 week after vaccination as compared by pre-vaccination levels. The $IgG_2$, the other subclass of immunoglobulin IgG which appears after $IgG_1$ production, also increased markedly only in the group of mice after exposure for 1 week to filtered air. The concentrations of both immunoglobulins returned to pre-vaccination levels after 2 and 4 weeks exposure to filtered air. The $IgG_1$ and $IgG_2$ concentrations in vaccinated mice exposed to 2 ppm $NO_2$ or to 0.5 ppm $NO_2$ with daily 2 ppm $NO_2$ peaks remained constant throughout the 4 week exposure and did not differ markedly from pre-vaccination levels.

In general, there appears that exposure to $NO_2$ influenced the immunoglobulin levels in mice. Results of long-term chronic exposures to $NO_2$ indicated an elevation of IgA, IgM, $IgG_1$, and

Table II-2

SERUM IMMUNOGLOBULIN CONCENTRATIONS IN VACCINATED MICE EXPOSED TO $NO_2$

| $NO_2$, ppm | Weeks Post-Vacc. | Immunoglobulin, mg/ml | | | |
|---|---|---|---|---|---|
| | | IgA | IgM | $IgG_1$ | $IgG_2$ |
| 0 | 0 | 0.413 | 0.094 | 1.18 | 3.17 |
| 0 | 1 | 0.368 | 0.338* | 3.12* | 5.26* |
| 0.5/2 | | 0.470↑ | 0.274↓ | 1.99 | 3.92 |
| 2 | | 0.366 | 0.286 | 1.25↓ | 2.44↓* |
| 0 | 2 | 0.416 | 0.255 | 1.11 | 2.77 |
| 0.5/2 | | 0.515* | 0.195 | 0.92 | 2.51* |
| 2 | | 0.455 | 0.248 | 1.02 | 2.45 |
| 0 | 4 | 0.400 | 0.255 | 1.27 | 3.41 |
| 0.5/2 | | 0.540* | 0.195 | 1.58 | 3.25 |
| 2 | | 0.880↑* | 0.252 | 1.55 | 3.41 |

Star indicates significant change from pre-vaccination level.

Arrow indicates significant change from control mice held in filtered air.

$IgG_2$ over the 28 week period. The 4 week exposure to $NO_2$ resulted in an elevation of IgA but an initial depression of IgM, $IgG_1$, and $IgG_2$. These differences are most likely due to the varying time of sampling whereby this experiment reflects the immunoglobulin concentration changes during the initial 4 weeks after vaccination. In addition, the animals used in the acute study were 2 to 4 weeks younger than at the initiation of the exposure than used in the chronic $NO_2$ study.

Infectious Challenge. Vaccinated mice and non-vaccinated mice injected with saline were challenged with airborne $A_2$/Taiwan influenza virus at 1, 2, and 4 weeks of exposure to $NO_2$. The mortality rates and lung lesion scores are summarized in Tables II-3 and II-4, respectively.

The vaccine afforded excellent protection against the infectious challenge under all exposure conditions and no significant differences were noted in mortality rates when mice held in $NO_2$ were compared to the control mice exposed to filtered air throughout the 4 week period. Similarily, the lung lesions scores were significantly lower in all vaccinated mice when compared to non-vaccinated mice. At 4 weeks after vaccination, the lung lesion scores were significantly less severe in mice exposed to $NO_2$ than those exposed to filtered air. These results are probably due to the very high efficacy of the vaccine or to an experimental variable. This was not observed in non-vaccinated mice nor in any other group of mice included in this experiment.

Mice surviving the virus challenge at 1, 2, and 4 weeks after vaccination were exsanguinated and the sera were assayed for HI antibody to determine the anamnestic response. The various groups of vaccinated mice showed a consistent anamnestic response to $A_2$/Taiwan influenza virus infection, with no effect of the $NO_2$ exposure being apparent.

Table II-3

MORTALITY RATE OF MICE CHALLENGED WITH
$A_2$/TAIWAN INFLUENZA VIRUS

| $NO_2$, ppm | Exposure to $NO_2$, Weeks | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | | | 2 | | | | 4 | | | |
| | Saline | | Vaccine | | Saline | | Vaccine | | Saline | | Vaccine | |
| | D/T | % | D/T | % | D/T | % | D/T | % | D/T | % | D/T | % |
| 0 | 15/19 | 79 | 0/20 | 0* | 9/20 | 45 | 1/16 | 6* | 12/20 | 60 | 2/12 | 17* |
| 0.5/2 | 18/20 | 90 | 0/20 | 0* | 13/20 | 65 | 1/20 | 5* | 12/18 | 67 | 0/20 | 0* |
| 2 | 13/20 | 65 | 1/20 | 5* | 12/20 | 60 | 0/20 | 0* | 14/20 | 70 | 0/20 | 0* |

* Significant differences between vaccinated and corresponding non-vaccinated mice.

Table II-4

LUNG LESIONS IN MICE CHALLENGED WITH $A_2$/TAIWAN INFLUENZA VIRUS

| $NO_2$, ppm | Lung Lesion Scores[a] | | | | | |
|---|---|---|---|---|---|---|
| | 1 Week | | 2 Weeks | | 4 Weeks | |
| | Saline | Vaccine | Saline | Vaccine | Saline | Vaccine |
| 0 | 4.40 | 0.85* | 3.70 | 0.87* | 3.80 | 1.60* |
| 0.5/2 | 4.85 | 0.70* | 4.25 | 0.80* | 3.83 | 0.15*+ |
| 2 | 4.20 | 1.40* | 3.80 | 0.25* | 4.15 | 0.26*+ |

(a) Each score represents the mean lung lesion score of 14 to 20 mice.

* Significant difference between vaccinated and corresponding non-vaccinated mice.

\+ Significant difference within vaccinated group when compared to mice held in filtered air.

Summary. Mice were vaccinated with $A_2$/Taiwan influenza vaccine and exposed for 4 weeks to either 2 ppm of $NO_2$, 0.5 ppm $NO_2$ with 1-hr peaks of 2 ppm $NO_2$ for 5 days per week, or to filtered air. The differences among the HI and SN antibody titers between control and experimental mice were not significant. There was an elevation in the levels of IgA, and an initial depression of IgM, $IgG_1$ and $IgG_2$ concentration. No statistical differences were noted in mortality rates or lung lesion scores when infected mice exposed to $NO_2$ were compared to infected mice held in filtered air. HI antibody titers in mice surviving virus challenge indicated that $NO_2$ exposure had no effect on the anamnestic response of vaccinated mice.

## III. EFFECT OF CHRONIC EXPOSURE TO $NO_2$ ON RESISTANCE TO K. PNEUMONIAE

### A. Introduction

Limited studies were conducted to determine changes in resistance to bacterial pneumonia associated in the fluctuation in concentrations of nitrogen dioxide ($NO_2$). Mice were held in a selected environment for 3 months and then re-exposed to the same environment or transferred to another environmental condition. At various time intervals the mice were challenged with Klebsiella pneumoniae, returned to the appropriate environmental condition and mortality rates were determined over a 14 day period.

### B. Materials and Methods

Animals. Four-week-old specific-pathogen-free male Swiss albino mice, CD-1 strain, were obtained from Charles River Laboratories. After a two-week quarantine, the mice were placed in the environmental chambers and held for two days before initiation of the exposures. During the exposures, the mice were removed from the chamber for one hour three times a week for maintenance. Clean cages were provided once a week and food and water were provided ad libitum.

Nitrogen Dioxide Exposure. To maintain the control and experimental mice under similar conditions, three identical aluminum-lined chambers (4 x 6 x 6.5 ft) were used for the exposures. Randomly selected mice were housed in suspended wire cages, which were periodically rotated to various positions on the cage racks. This assured a thorough and unbiased exposure to the experimental environment. A minute amount of $NO_2$ was continuously passed from a cylinder through a stainless steel tube into a glass mixing vessel where it was diluted and mixed with charcoal-filtered ambient air. The mixture was then passed into the $NO_2$ exposure chambers at a rate of 20 changes per hour.

The same air flow pattern was maintained in the control chamber where the charcoal-filtered ambient air was used. To verify the homogeneity of $NO_2$, air samples were taken from different sections of the chambers and the $NO_2$ concentration determined and calculated by the Saltzman method. A Mast $NO_2$ gas analyzer was used for continuous monitoring. The mean temperature in the chambers was 24° ± 1°C.

Klebsiella pneumoniae. Bacterial challenge was made with mouse-adapted K. pneumoniae type A, strain A-D. To prepare the stock culture K. pneumoniae was passaged in mice, isolated from the heart, and grown in trypticase soy broth for 18 hr at 37°C in static culture. The bacteria were then streaked onto blood agar slants, incubated for 18 hr at 37°C, and held at 4°C until used. For dissemination, bacteria from the slants were grown in trypticase soy broth at 37°C for 18 hr in static culture. The concentration of the culture was adjusted by measurement of transmission on a Spectronic 20 densitometer at 440 μm. Appropriate dilutions of the culture were used for dissemination to obtain approximately 20% mortality in control group of mice.

Aerosol Challenge. Infectious respiratory challenge was conducted in a 350-liter plastic aerosol chamber (60 x 60 x 95 cm) installed within a microbiological safety cabinet. A University of Chicago Toxicity Laboratory type atomizer was used to produce the bacterial aerosol with a majority of <5μ-mass-median-diameter particles. The microbial suspensions were fed to the atomizer by a 50-ml syringe activated by a motor-driven piston delivering the suspension at a rate of 0.4 ml/min. Filtered air was supplied to primary and secondary inlets of the atomizer at a flow rate of 33 liters/min. The chamber was maintained at 78 ± 6% RH and 24° ± 1°C.

The aerosol was sampled with an all-glass impinger (AGI-30) containing PBS with 0.2% bovine serum albumin as a collecting fluid. The inhaled dose was estimated on the basis of the concentration of K. pneumoniae per liter of air, respiratory

minute volume of the mice, and the duration of exposure to the aerosol.

For the infectious challenge, groups of mice were placed in the chamber and exposed to the aerosol for 5 to 6 min. After the challenge, the mice were air-washed for 10 min, removed from the aerosol chamber and held for 14 days in filter-capped cages in a clean-air, isolated animal room.

Statistical Analysis. As appropriate, the experimental results were subjected to statistical analysis and the significance of the observed differences was reported at the $\leq$5% probability level. The significance of the differences in mortality rates was determined by the normal approximation with correction for cintinuity (K. S. Brownlee, Statistical Theory and Methodology in Science and Engineering, p. 121, 1960, John Wiley and Sons, New York). This method is equivalent to the chi-square test applied with a 2 x 2 contingency table, except for the use of corrections for continuity.

## C. Results

Mortality rates were determined in mice continuously exposed for up to 9 months to either 2 ppm $NO_2$, 0.5 ppm $NO_2$ with daily 1-hr peaks of 2 ppm $NO_2$, or filtered air. At various time intervals during the exposure groups of 20 mice were challenged with airborne K. pneumoniae and mortality rates were observed over a 14-day holding period. The results of the experiment summarized in Table III-1, indicate that there was an increase in mortality rates among mice exposed to 2 ppm $NO_2$ and to a lesser degree among those exposed to 0.5 ppm $NO_2$ with daily 1-hr peaks of 2 ppm $NO_2$. Because of the very limited number of mice available in this study, replicate experiments were not performed. Thus the statistical significance of the differences could be ascertained only for groups exposed to $NO_2$ for eight months. However, as

Table III-1

PERCENT MORTALITY IN MICE EXPOSED TO $NO_2$ AND CHALLENGE WITH K. PNEUMONIAE

| $NO_2$, ppm | $NO_2$ Exposure, Months | | | |
|---|---|---|---|---|
| | 6 | 7 | 8 | 9 |
| 0 | 5 | 65 | 25 | 15 |
| 0.5/2 | 23 | 50 | 60* | 15 |
| 2 | 26 | 70 | 75* | 25 |

* Significant change when compared to mice exposed to filtered air (0 ppm $NO_2$).

previously reported from our laboratories chronic exposure to 0.5 ppm $NO_2$ reduced the resistance of mice to subsequent challenge with airborne K. pneumoniae. The limited experiments summarized in this report confirmed these observations.

Mice exposed for 3 months to 0.5 ppm $NO_2$ with 1-hr peaks of 2 ppm $NO_2$ and to 2 ppm $NO_2$ were held in filtered air for an additional 6 months. During this recovery period groups of 20 mice were challenged with airborne K. pneumoniae and the mortalities were observed during a 14-day holding period. The results summarized in Table III-2 indicate no marked differences in mortality rates, especially when compared to mice exposed to filtered air only.

Table III-2

PERCENT MORTALITY IN MICE CHALLENGED WITH *K. PNEUMONIAE* DURING RECOVERY FROM EXPOSURE TO $NO_2$

| 3-Months Exposure $NO_2$, ppm | Filtered Air Exposure, Months | | | |
|---|---|---|---|---|
| | 3 | 4 | 5 | 6 |
| 0 | 5 | 65 | 25 | 15 |
| 0.5/2 | 15 | 40 | 50 | 25 |
| 2 | 5 | 50 | 55 | 15 |

## IV. EFFECTS OF MANGANESE ON RESISTANCE TO RESPIRATORY INFECTION

### A. Introduction

Studies were conducted to determine the effect of acute exposures of airborne manganese oxide (MnO) or manganese dioxide ($MnO_2$) on resistance of mice to bacterial or viral respiratory infections. Initially, exploratory experiments were conducted to develop aerosolization techniques by means of the Wright Dust Feeder. Mice were exposed to airborne manganese oxide (MnO) or manganese dioxide ($MnO_2$) for various time periods, killed, and the lungs were examined microscopically to observe the deposition and retention of particles.

Thereafter groups of mice were exposed daily for 3 hrs up to 4 days, to the manganese compounds. The mice were challenged with influenza virus before exposure and with K. pneumoniae following the exposure to manganese.

### B. Materials and Methods

Animals. Four week-old specific-pathogen-free male Swiss albino mice, CD-1 strain, were obtained from Charles River Laboratories. After a two-week quarantine period, the mice were used for the exposures. Throughout the experiments clean cages were provided once a week and food and water were provided ad libitum.

Influenza Virus. Mouse-adapted influenza A/PR/8 virus was passaged several times in mice and 20% lung suspension of the virus was used for all infectious challenges. Prior to use, the virus was identified by use of specific antiserum obtained from the National Institutes of Health.

Klebsiella pneumoniae. To prepare the stock culture, K. pneumoniae type A, strain A-D were passaged in mice, isolated from the heart, and grown in trypticase soy broth for 18 hr at 37°C in static culture. The bacteria were then streaked onto blood agar slants, incubated for 18 hr at 37°C, and held at 4°C until used. For dissemination, bacteria from the slants were grown in trypticase soy broth at 37°C for 18 hr in static culture. The concentration of the culture was adjusted by measurement of transmission on a Spectronic 20 densitometer at 440 μm. Appropriate dilutions of the culture were used for dissemination to obtain approximately 20% mortality in control group of mice.

Aerosol Challenge. Infectious respiratory challenge was conducted in a 350-liter plastic aerosol chamber (60 x 60 x 95 cm) installed within a microbiological safety cabinet. A University of Chicago Toxicity Laboratory type atomizer was used to produce the microbial aerosol with a majority of <5μ-mass-median-diameter particles. The microbial suspensions were fed to the atomizer by a 50-ml syringe activated by a motor-driven piston delivering the suspension at a rate of 0.4 ml/min. Filtered air was supplied to primary and secondary inlets of the atomizer at a flow rate of 33 liters/min. The chamber was maintained at 78 ± 6% RH and 24 ± 1°C.

The aerosol was sampled with an all-glass impinger (AGI-30) containing PBS with 0.2% bovine serum albumin as a collecting fluid. The inhaled dose was estimated on the basis of the concentration of the microorganisms per liter of air, respiratory minute volume of the mice, and the duration of exposure to the aerosol.

For the infectious challenge, groups of mice were placed in the chamber and exposed to the aerosol for 5 to 6 min. After the challenge, the mice were air-washed for 10 min, removed from the aerosol chamber and held for 14 days in filter-capped cages in a clean-air, isolated animal room.

Scoring Pulmonary Lesions. The extent of pulmonary lesions after challenge with influenza virus was expressed as the percentage of the total lung consolidated. A score of 1 represented 25% lung consolidation, 2 = 50%, 3 = 75%, 4 = 100%, and a score of 5 represented animals that died during the experiment.

Exposure to Manganese. Mice were placed in a plexiglass chamber (120 x 60 x 60 cm; 430 liter capacity) and exposed for 3 hr to MnO or $MnO_2$ aerosols produced by the Wright Dust Feed Mechanism (L. Adams Ltd., London, England). The amounts of MnO or $MnO_2$ disseminated in the chamber over the 3 hr period were 50 and 76 g, resulting in an aerosol concentration of approximately 110 μg MnO/ml and 168 μg $MnO_2$/ml of air. To ensure uniform exposure to aerosol, randomly selected mice were housed in wire cages, which were rotated at least once during the 3-hr exposure to vary the position within the chamber.

During the aerosol exposure, microscopic slides were placed in horizontal positions in the chamber for time periods ranging from 15 to 60 sec. The slides were viewed at 1000x magnification and 100 particles were counted to determine the percentage of particles equal to or less then 5μ in diameter. Approximately 5% of the MnO particles were $\leq 5\mu$ diameter, whereas 75% of the $MnO_2$ particles were in this size range.

Statistical Analysis. As appropriate, the experimental results were subjected to statistical analysis and the significance of the observed differences was reported at the $\leq 5\%$ probability level. The significance of the differences in mortality rates was determined by the normal approximation with correction for continuity (K. S. Brownlee, Statistical Theory and Methodology in Science and Engineering, p. 121, 1960, John Wiley and Sons, New York). This method is equivalent to the chi-square test applied with a 2 x 2 contingency table, except for the use of corrections for continuity. The Student t-test was used for statistical analysis of the lung lesion scores.

The relative mean survival rate (RMSR) was calculated according to the following equation

$$RMSR_d = \frac{(\Sigma A \times B) + (d \times L)}{n}$$

Where A is the last day on which any individual mouse was alive; B is the number of mice surviving A days; d is the last day of the experiment (14 or 15); L is the number of mice which were alive on day d; and n is the initial number of mice in the experimental group. The significance of the differences in the RMSR values between the experimental and control groups was assessed by the Student t-test (2-tailed).

## C. Results

MnO Exposure and Challenge with Influenza Virus. Mice were exposed to MnO for 3 hr daily, with the first exposure occurring 24 hr after the challenge with influenza virus. Since approximately 95% of the MnO particles were larger than 5μ in diameter, groups of mice were sacrificed to determine whether MnO particles were present in the lungs. Following three 3-hr exposures, MnO particles were not seen in the lung upon examination in transmitted or polarized light microscope. After four 3-hr exposures, a small number of MnO particles was scattered throughout the lung tissue. Nevertheless, as shown in Table IV-1 increased mortality rates were observed among mice challenged with the virus and exposed to MnO.

$MnO_2$ Exposures and Challenged with K. pneumoniae. During the initial experiments, lungs were excised from the mice immediately after each exposure to $MnO_2$. Microscopic observation of the lungs revealed that $MnO_2$ particles were present after a single 3 hr exposure and the number of particles increased with the number of the 3-hr exposures to $MnO_2$. At all time periods, an acute to subacute septal infiltrate was present, with slight septal edema

Table IV-1

MORTALITY RATE AMONG MICE CHALLENGED WITH INFLUENZA VIRUS AND EXPOSED TO MnO AEROSOL

| Experimental Condition | Mortality | |
|---|---|---|
| | Dead/Total | % |
| MnO (3)[a] | 1/20 | 5 |
| MnO (4) | 2/30 | 7 |
| Virus | 6/40 | 15 |
| Virus → MnO (3)[b] | 11/30 | 37* |
| Virus → MnO (4) | 9/30 | 30 |

[a] Number of daily 3-hr MnO exposures.

[b] First MnO exposure 24 hr after infectious challenge.

* Significant difference when compared with control mice challenged with influenza virus.

and congestion. The extent of these changes was greatest in lungs of mice after the fourth 3-hr exposure to $MnO_2$.

Significantly enhanced mortality rates and reduced relative mean survival rates (RMSR) were observed following one or more $MnO_2$ exposures and challenged with airborne K. pneumoniae (Table IV-2). When the interval between the $MnO_2$ exposure and the infectious challenge was 1 hr increased mortality rates were observed among mice exposed to manganese for three and four daily 3-hr periods. A concurrent reduction in mean survival time was also seen. Increase in interval between exposure to $MnO_2$ and the infectious challenge to 5 hr resulted in more pronounced increases in mortality rates and decreases in survival time which were observed after a single exposure to $MnO_2$.

$MnO_2$ Exposure and Challenge with Influenza Virus. Mice were challenged with the influenza virus 24 and 48 hr before exposure to aerosols of $MnO_2$. As seen in Table IV-3 at 24 hr the only significant increase in mortality and mean lung lesion scores were observed following a single 3-hr exposure to $MnO_2$. Repeated exposures to $MnO_2$ also resulted in enhanced death rates but the statistical significance of the differences could not be ascertained. When the exposure to $MnO_2$ was delayed to 48 hr after the infectious challenge significant mortality and lung lesions score increases occurred after both one and two 3-hr exposures to $MnO_2$. The third 3-hr exposure to $MnO_2$ also resulted in increased mortality rates and lesion scores but the differences were not significant.

Results of the exploratory study indicate that a 3-hr exposure to MnO or $MnO_2$ aerosols results in increased susceptibility to bacterial pneumonia and influenza infection. The increase in susceptibility was demonstrated by increased mortality rates and decreased mean survival time after challenge with airborne K. pneumoniae and increased mortality rates and lung lesion scores after challenge with airborne influenza virus.

Table IV-2

MORTALITY AND RELATIVE MEAN SURVIVAL RATE (RMSR) OF MICE EXPOSED TO $MnO_2$ AEROSOL AND CHALLENGED WITH K. PNEUMONIAE

| Experimental Condition | Interval Between $MnO_2$ Exposure and K. pneumoniae Challenge: 1 hr Mortality D/T | 1 hr Mortality % | 1 hr RMSR Days | 5 hr Mortality D/T | 5 hr Mortality % | 5 hr RMSR Days |
|---|---|---|---|---|---|---|
| $MnO_2$ (1)[a] | 0/25 | 0 | 15.0 | 0/15 | 0 | 14.0 |
| $MnO_2$ (2) | 0/25 | 0 | 15.0 | 0/15 | 0 | 14.0 |
| $MnO_2$ (3) | 0/25 | 0 | 15.0 | 0/15 | 0 | 14.0 |
| $MnO_2$ (4) | 0/25 | 0 | 15.0 | 0/15 | 0 | 14.0 |
| K. pneumoniae | 8/31 | 26 | 13.7 | 20/61 | 33 | 10.8 |
| $MnO_2$ (1) → K. pneumoniae | 9/27 | 33 | 10.6 | 35/60 | 58* | 7.0* |
| $MnO_2$ (2) → K. pneumoniae | 5/25 | 20 | 12.4 | 36/60 | 60* | 7.1* |
| $MnO_2$ (3) → K. pneumoniae | 12/25 | 48 | 9.4* | 31/60 | 52* | 7.9* |
| $MnO_2$ (4) → K. pneumoniae | 12/25 | 48 | 9.5* | 29/60 | 48 | 8.7* |

[a] Number of daily 3-hr $MnO_2$ exposures.

* Significant difference when compared to control mice challenged with K. pneumoniae.

Table IV-3

MORTALITY RATE AMONG MICE CHALLENGED WITH INFLUENZA VIRUS AND EXPOSED TO $MnO_2$ AEROSOL

| Experimental Condition | Interval Between Virus Challenge and $MnO_2$ Exposure: 24 hr Mortality D/T | 24 hr Mortality % | 24 hr Lung Lesions | 48 hr Mortality D/T | 48 hr Mortality % | 48 hr Lung Lesions |
|---|---|---|---|---|---|---|
| $MnO_2$ (1)[a] | 0/30 | 0 | 0.4 | 0/5 | | |
| $MnO_2$ (2) | 0/30 | 0 | 0 | 0/5 | | |
| $MnO_2$ (3) | 0/30 | 0 | 0 | 0/5 | | |
| $MnO_2$ (4) | 0/30 | 0 | 0 | | | |
| Influenza Virus | 6/44 | 14 | 1.63 | 9/30 | 30 | 2.57 |
| Virus → $MnO_2$ (1) | 13/34 | 38* | 2.97* | 21/30 | 70* | 4.03* |
| Virus → $MnO_2$ (2) | 7/34 | 21 | 1.79 | 19/30 | 63* | 3.77* |
| Virus → $MnO_2$ (3) | 9/45 | 20 | 2.02 | 13.30 | 43 | 3.17 |
| Virus → $MnO_2$ (4) | 11/47 | 23 | 2.49 | - | | - |

[a] Number of daily 3-hr $MnO_2$ exposures.

* Significant difference when compared to control mice challenged with influenza virus.